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## Regeneration of irradiated salivary glands by stem cell therapy

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## CHAPTER 6

### **Palifermin Prevents Radiation Damage to Salivary Glands by Expansion of the Stem/Progenitor Pool**

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**Submitted**



## ABSTRACT

Irradiation of salivary glands during radiotherapy treatment of patients suffering from head and neck cancer evokes persistent hyposalivation. This results from depletion of stem cells rendering the gland incapable to replenish saliva producing acinar cells. This study aims to investigate whether it is possible to expand the salivary gland stem/progenitor cell population, thereby preventing acinar cell depletion and subsequent gland dysfunction after irradiation.

To induce cell proliferation,  $\Delta$ N23-KGF (Keratinocyte Growth Factor, Palifermin) was administered to C57BL/6 mice for 4 days before and/or after local irradiation of salivary glands. Salivary gland vitality was quantified by *in vivo* saliva flow rates, morphological measurements, and a newly developed *in vitro* salisphere progenitor/stem cell assay. Irradiation of salivary glands led to a pronounced reduction in the tissues stem cells, resulting in severe hyposalivation and a reduced number of acinar cells.  $\Delta$ N23-KGF treatment for 4 days prior to irradiation indeed induced salivary gland stem/progenitor cell proliferation, increasing the stem- and progenitor cell pool. This did not change the relative radiation sensitivity of the stem/progenitor cells, but as a consequence an absolute higher number of stem/progenitor cells and acinar cells survived after radiation. Post-irradiation treatment with  $\Delta$ N23-KGF also improved gland function, and this effect was much more pronounced in  $\Delta$ N23-KGF pre-treated animals. Post-treatment with  $\Delta$ N23-KGF seemed to act through accelerated expansion of the pool of progenitor/stem cells that survived the irradiation treatment.

Overall, our data indicate that  $\Delta$ N23-KGF is a promising drug to enhance the number of salivary gland progenitor/stem cells and consequently prevent radiation-induced hyposalivation.

## INTRODUCTION

Yearly, more than 500,000 new patients are diagnosed with head and neck cancer world-wide <sup>1,317</sup>. Radiotherapy, either alone or in combination with surgery and chemotherapy, is often applied as treatment of these patients. The radiation dose by which the tumor can be treated is limited by the sensitivity of surrounding normal tissues within the field of radiation. For head and neck cancers, even with the most optimal radiation schedule, salivary glands are one of these tissues at risk. Progressive loss of function may occur already within the first weeks of radiotherapy, and can persist for life <sup>318</sup>. Radiation-induced salivary gland dysfunction may cause oral dryness, dental caries, hampered speech and xerostomia (= dry mouth syndrome), which collectively severely limit the quality of life of the patients <sup>3,4</sup>.

The delayed loss of gland function after radiation is thought to be due to a loss of stem cells that are no longer able to replenish aged saliva-producing acinar cells <sup>16</sup>. In normal salivary glands, the ductal system that include excretory, striated, and intercalated ducts, biochemically modifies and transports saliva, produced by acinar cells, into the oral cavity. This ductal system also contains the tissue stem/progenitor cells <sup>128,129</sup>. Proliferation and differentiation of these primitive cells within the ducts maintains homeostasis of the acinar cells. In theory, expansion of the salivary gland stem/progenitor cell population may prevent acinar cell depletion and subsequent gland dysfunction after radiation.

Although the effect of  $\Delta$ N23-KGF (Keratinocyte Growth Factor, FGF-7, Palifermin) on the salivary gland has not been studied,  $\Delta$ N23-KGF has been shown to ameliorate radiation-induced damage in a variety of other tissues, such as lung <sup>319-322</sup>, gut <sup>323,324</sup>, tongue <sup>325</sup>, and oral mucosa <sup>326</sup>. The mechanism is believed to result from either stimulation of proliferation <sup>324,327-331</sup>, direct radioprotection <sup>332</sup>, and/or stimulation of cell motility <sup>333</sup>.

In this study, several  $\Delta$ N23-KGF treatment schedules were evaluated for their efficacy to reduce salivary gland morphology and function after irradiation in mice. In addition, effects were evaluated using a recently developed *in vitro* salivary gland stem/progenitor assay <sup>220</sup>. It was found that  $\Delta$ N23-KGF administration before and after irradiation of salivary glands induced a long-term expansion of duct stem/progenitor cells, which elicited a short- and long-term maintenance of the acinar cell compartment, resulting in permanent preservation of the salivary gland function after irradiation.

RESULTS

**ΔN23-KGF PREVENTS RADIATION-INDUCED SALIVARY GLAND DYSFUNCTION**

To investigate whether administration of ΔN23-KGF could reduce radiation toxicity to salivary glands, mice receiving local salivary gland irradiation (15 Gy, IR) were treated with ΔN23-KGF either 4 times before (K-IR) or after irradiation (IR-K), or for a 7 day schedule with irradiation in the middle of the treatment (K-IR-K) (Fig. 1). As control groups, mice that were only irradiated and mice that received only ΔN23-KGF were included. Salivary gland function measurements (30, 60 and 90 days post-irradiation) showed that saliva production in non-irradiated mice that were treated with ΔN23-KGF for 4 or 7 days (4D and 7D K) was not significantly different from untreated animals (Fig. 2A), albeit with some gland hypertrophy as indicated by an increase in wet weight of the submandibular glands (Suppl. Fig. 1). As expected, irradiated mice that did not receive ΔN23-KGF developed severe hyposalivation (Fig. 2A). In contrast, ΔN23-KGF treatment, irrespective of the treatment schedule, resulted in significantly more saliva production in irradiated mice than non-treated animals. ΔN23-KGF treatment before radiation resulted in a delayed onset of radiation-induced reduction in saliva flow rate, whereas ΔN23-KGF treatment after radiation was least effective. However, when ΔN23-KGF was administered both prior to and after radiation, saliva production was best preserved (Fig. 2A). To establish potential dosis dependency of a K-IR-K treatment, saliva flow rate was measured at 30 days after graded doses of irradiation (Fig. 2B). Indeed, a dose dependent reduction in flow rate was observed, however with a virtually identical slope as irradiation alone, indicating that the effect of ΔN23-KGF treatment before and after irradiation is not radiation dose dependent.

Significantly higher wet submandibular gland weights were observed 3 months after a 4 or 7 day ΔN23-KGF treatment, when compared to untreated animals (Suppl. Fig. 1). Ninety days post-irradiation submandibular gland weight decreased to 58% of untreated controls. In agreement with the flow rate data, all ΔN23-KGF treatment schedules had significant beneficial effects on submandibular gland weight, and gland weight correlated with saliva production, suggesting that ΔN23-KGF exerts protective effects on gland cells before and after irradiation.

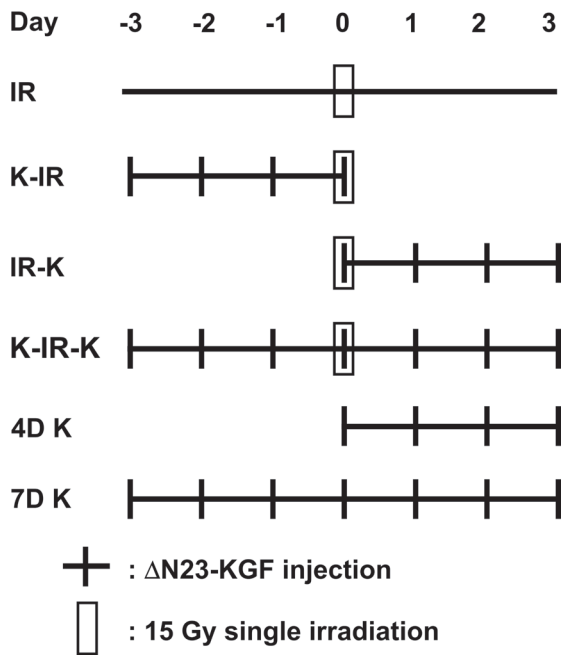
**TROPIC EFFECT OF ΔN23-KGF ON ACINAR CELLS**

To investigate which cells are responsible for the protective effect of ΔN23-KGF, submandibular glands from ΔN23-KGF-treated irradiated and non-irradiated mice were examined (Fig. 3).

A normal salivary gland consists for about 60% of PAS<sup>+</sup> mucin containing acinar cells grouped in acini (Fig. 3A, purple cells, arrows) connected to the ductal compartment (Fig. 3A, blue cells, arrowheads) comprising 3 different cell types; intercalated (ID), striated (SD) and excretory duct (ED) cells which are collectively responsible for the modification and transport of the saliva produced by the acinar cells<sup>277</sup>.

ΔN23-KGF treatment for 4 or 7 days (4D K and 7D K) caused a rapid (24 hours after last injection) but modest increase in acinar cell surface area (10%) (data not shown) which almost normalized in time (90 days post-treatment) (Fig. 3B,G).

Massive acinar cell depletion (Fig. 3C, arrows and 3G) and fibrotic cell deposition (Fig. 3C, asterisks), hallmarks of late irradiation-damage, were clearly visible 90 days post-irradiation. In contrast, post-irradiation treatment with ΔN23-KGF (Fig. 3D,G) reduced acinar cell loss, whereas pre-treatment largely (Fig. 3E,G) and 'pre- plus post-' treatment (Fig. 3F,G) almost completely abrogated the net loss of acinar cells.

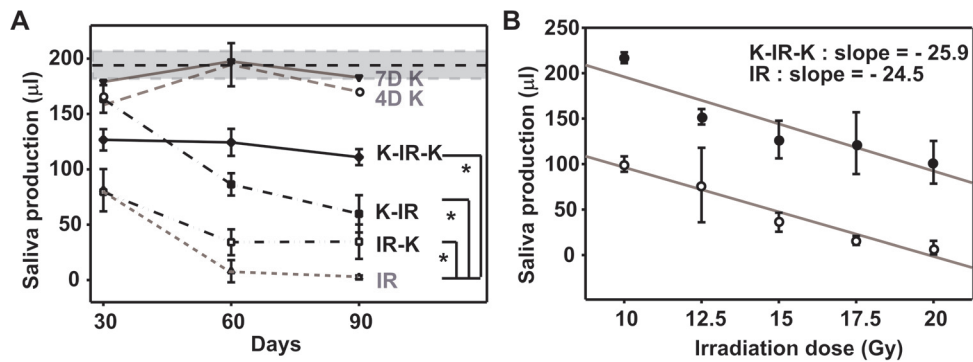


**FIGURE 1. SCHEMATIC REPRESENTATION OF EXPERIMENTAL SETUP.** Local 15 Gy salivary gland irradiation (IR) was given before (K-IR) or after (IR-K) a 4 days ΔN23-KGF treatment (5 mg/kg/day), or on the 4th day of a 7 days ΔN23-KGF treatment (K-IR-K). Mice in the 4D or 7D KGF group only received daily ΔN23-KGF injections for 4 or 7 days, respectively.

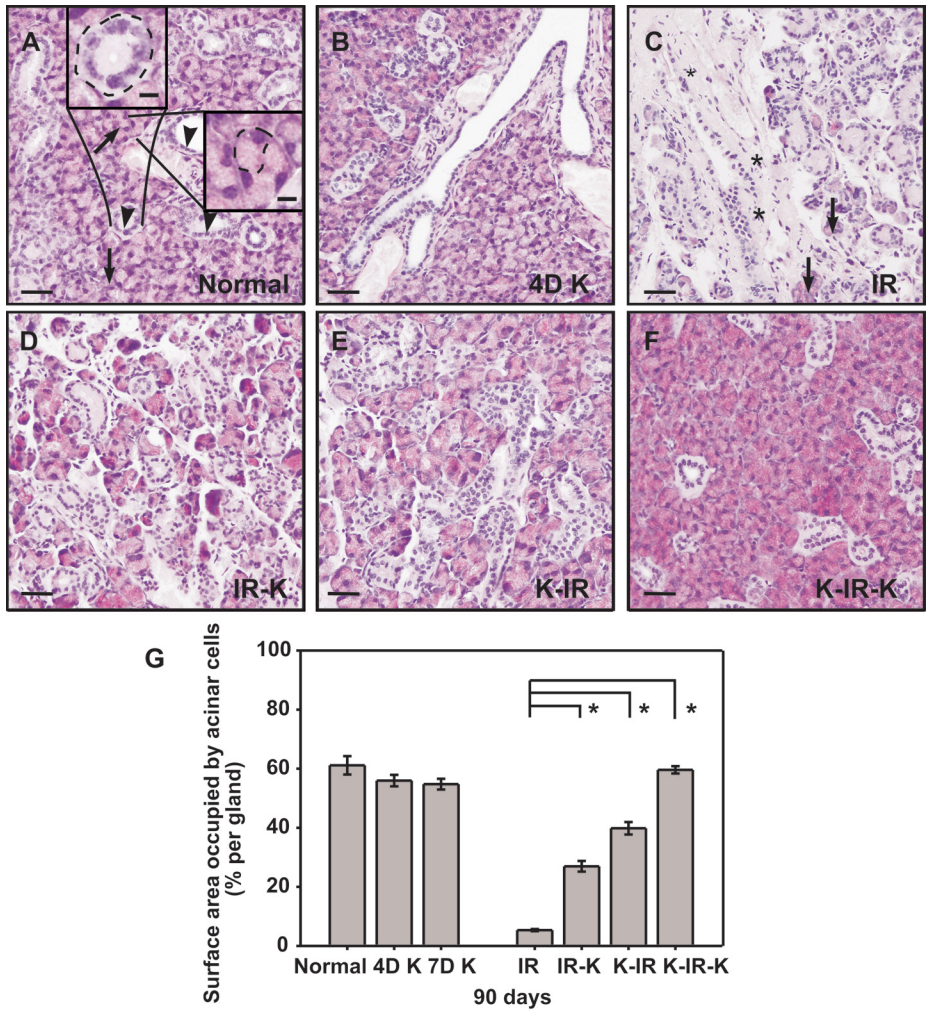


ΔN23-KGF ENHANCES STEM/PROGENITOR CELL NUMBERS

To assess whether the protective effect of ΔN23-KGF is due to a proliferative effect on acinar cells, *in vivo* BrdU incorporation in submandibular glands from animals treated with ΔN23-KGF for 4 days was investigated. Whereas glands from normal mice hardly showed any proliferating cells, large numbers of BrdU<sup>+</sup> acinar cells were observed in salivary glands of ΔN23-KGF-treated animals 24 hours after the last treatment (Fig. 4A). Similar effects of ΔN23-KGF on the proliferation of oral mucosa were shown before by Borges *et al.*<sup>328</sup>. Additionally, BrdU incorporation was clearly present in intercalated (ID, arrowheads) and excretory duct cells (ED, arrows), indicating that also these cell types were stimulated to proliferate. Remarkably, however, the receptor for ΔN23-KGF, FGFR2-III-b, was exclusively expressed on intercalated (Fig. 4B, arrowheads) and excretory duct cells (Fig. 4B, arrows), and not on acinar cells. In addition to the 10% increase noticed after 24hrs in the 4D KGF treated mice, this implies that the increase in proliferation of acinar cells likely originated from a direct stimulatory effect of ΔN23-KGF on intercalated and/or excretory duct cells which subsequently differentiate into acinar cells. To test this, the surface area occupied by these different ductal cell types, as a reflexion of cell number, was evaluated after a 4 day exposure of ΔN23-KGF. Twenty-four hours after the last ΔN23-KGF injection, an increase in surface area of excretory (2.8 fold) and intercalated ducts cells (1.8 fold) in these glands was observed (Fig. 4C), reflecting their high proliferation rate.



**FIGURE 2. ΔN23-KGF TREATMENT AFFECTS GLAND FUNCTION.** (A) Saliva flow rates were measured 30, 60, and 90 days post-radiation. IR-K, K-IR and K-IR-K (see Figure 1) treated mice produced significant more saliva compared to the untreated irradiated mice (IR). Saliva flow rates of 4D K and 7D K treated mice did not significantly differ from normal mice. (B) Saliva flow rates were measured of IR and K-IR-K treated mice 30 days post-irradiation. No change in slope could be detected. For all data a minimum of 3 mice were used. \*, *P* < 0.05. Error bars, SEM.



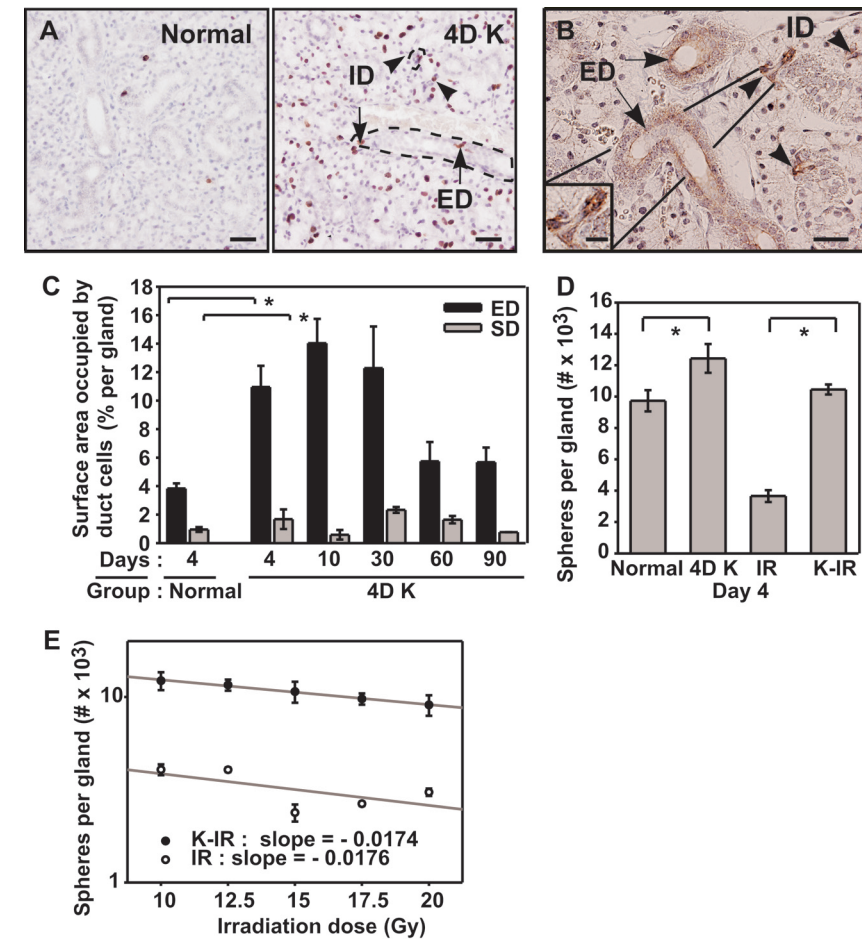
**FIGURE 3. THE EFFECT OF ΔN23-KGF ON ACINAR CELL NUMBER.** (A-F) Salivary glands of different groups (Normal, IR, 4D K, K-IR, IR-K, and K-IR-K, see Figure 1) were evaluated by PAS staining 90 days post-irradiation. Arrows represent acinar cells, arrowheads duct cells, and asterisks hallmark fibrosis. Enlarged pictures represent duct and acinar cells (dotted line in insets). (G) 90 days post-irradiation, glands of K-IR, IR-K, and K-IR-K treated mice contained significant more acinar cells than IR treated ones. \*, *P* < 0.05. Scale bar = 50 μm, inset = 20 μm, *N* ≥ 3, depicted as mean ± SEM.

After ninety days, the surface area occupied by excretory duct cells remained somewhat elevated, whereas that of other cell types was back to baseline values. The transient enhancement of the number of intercalated and especially excretory duct cells shortly after treatment indeed indicates that the increase in proliferation of acinar cells originates from dividing and subsequently differentiating intercalated ducts cells. It also suggests that  $\Delta$ N23-KGF might expand the number of stem/population cells known to reside in the ducts<sup>128</sup>.

To further substantiate the idea of expanding the pool of stem/progenitor cells, we cultured (sali)-spheres from salivary gland stem/progenitor cells<sup>220</sup>, similar to mammospheres<sup>334</sup> and neurospheres<sup>98</sup>. Interestingly, significant more salispheres were formed from submandibular glands of mice treated with  $\Delta$ N23-KGF for 4 days compared to untreated animals (Fig. 4D), demonstrating that KGF indeed induced the expansion of salivary gland salisphere forming cells. As expected, irradiation (15 Gy) resulted in a pronounced reduction in the number of salispheres formed in culture. In the  $\Delta$ N23-KGF pre-treated animals, the remaining number of salisphere forming cells that were recovered from the glands after 15 Gy was far higher and almost equal to the number recovered from glands from untreated controls (Fig. 4D). Salispheres cultured from untreated animals contained ~5 % c-Kit<sup>+</sup> cells, a percentage that did not change after IR and/or  $\Delta$ N23-KGF treatment (data not shown). This indicates that  $\Delta$ N23-KGF treatment results in a net increase in salivary gland stem/progenitor cells (expansion), and thus the absolute number or remaining salispheres forming cells after radiation is increased.

However, it has also been suggested that  $\Delta$ N23-KGF may be radio-protective for certain types of cells<sup>332,335-337</sup>, which would imply that also the relative loss of salivary gland stem/progenitor cells may be reduced after  $\Delta$ N23-KGF pre-treatment. In fact, our 15 Gy data (Fig. 4D) indeed suggested that the relative loss of salispheres forming cells may be reduced after  $\Delta$ N23-KGF pre-treatment. To test whether this effect is indeed significant, glands of mice treated with  $\Delta$ N23-KGF were subsequently irradiated with graded doses (10, 12.5, 15, 17.5 and 20 Gy), and the number of salispheres that were formed *in vitro* 24hrs after last  $\Delta$ N23-KGF injection was calculated (Fig. 4E). Indeed, a dose dependent decrease in salisphere forming cells was observed. At all doses, the number of salispheres from irradiated glands was significantly higher after  $\Delta$ N23-KGF-treatment than after irradiation alone. However, the relative radiation-induced decrease in sphere formation after an  $\Delta$ N23-KGF pre-treatment was equal over all doses, and the slopes of these curves were virtually the same (Fig. 4E). This suggests that  $\Delta$ N23-KGF has no effect on the intrinsic radio-sensitivity of these primitive cells.

Taken together, these data show that  $\Delta$ N23-KGF increases resistance of salivary glands to irradiation by increasing the endogenous stem cell compartment, resulting in larger absolute number of surviving stem cells after irradiation.



**FIGURE 4.  $\Delta$ N23-KGF INCREASES PROGENITOR/STEM CELLS AFTER RADIATION.** (A) Untreated, normal glands and glands from 4D K (see Figure 1) treated mice were evaluated for BrdU incorporation presence 24 hours after last  $\Delta$ N23-KGF/BrdU administration. BrdU staining (brown) was present in excretory duct (ED) cells (arrows) and intercalated duct (ID) cells (arrowheads). (B) Immunostaining for  $\Delta$ N23-KGF receptor (brown) revealed its expression by ID (arrowheads, inset) and ED cells (arrows). (C) Significant increased surface areas occupied by ED and ID cells in 4D K treated mice compared to normal glands at day 4. (D) The number of salispheres formed in culture from glands of Normal, 4D K, IR and K-IR treated mice sacrificed on day 4 showed that significantly more salispheres were detected after  $\Delta$ N23-KGF treatment compared to untreated groups. (E) Salispheres were counted from IR and K-IR treated mice which received different radiation doses. Significantly more salispheres were recovered from glands in the K-IR group when compared to IR. However, the irradiation dose-reponse curve (slope) was not altered by KGF. For all data in figure C-E, a minimum of 3 mice were used. \*,  $P < 0.05$ . Error bars, SEM. Scale bar = 50  $\mu$ m, inset = 20  $\mu$ m.



## POST-IRRADIATION $\Delta$ N23-KGF TREATMENT EXPANDS THE NUMBER OF RADIATION-SURVIVING STEM CELLS

If the protection of  $\Delta$ N23-KGF pre-treatment is due to an increment in the pool of progenitor/stem cells, the question arises why the post-treatment with  $\Delta$ N23-KGF enhances gland recovery, especially in glands that were also pre-treated with  $\Delta$ N23-KGF (Fig. 2-3). To selectively investigate the effects of post-radiation  $\Delta$ N23-KGF, we analyzed the glands of animals 24 hours after the last injection for proliferative cells using BrdU labelling. In glands that were irradiated without  $\Delta$ N23-KGF, BrdU positive cells were totally absent (not shown), but post-treatment with  $\Delta$ N23-KGF revealed BrdU positive cells in ID (Fig. 5A) and ED cells (not in figure). In parallel, the surface area of these duct cells was higher at 10 days and even at 90 days after irradiation for the animals post-treated with  $\Delta$ N23-KGF (Fig. 5B). In agreement with this, a post-irradiation  $\Delta$ N23-KGF treatment induced almost a doubling in the number of salispheres that could be isolated from glands 4 days after irradiation when compared to untreated glands (Fig. 5C). Similar, albeit more pronounced, results were obtained after a  $\Delta$ N23-KGF 'pre- and post'-irradiation treatment schedule (data not shown). Summarizing, whilst  $\Delta$ N23-KGF pre-treatment enhances absolute salivary gland cell numbers before radiation, post-treatment accelerates the expansion of these surviving progenitor/stem cells pool. The latter effect is obviously more pronounced when there are more remaining progenitor/stem cells, explaining why post-treatment with  $\Delta$ N23-KGF is specifically effective in sparing gland function in  $\Delta$ N23-KGF-pre-treated animals.

## DISCUSSION

This study demonstrates that expansion and activation of stem/progenitor cells by administration of  $\Delta$ N23-KGF prior to and after irradiation of the salivary glands yielded an almost normal saliva secretion and a long-term preservation of all submandibular gland cell types.

Several studies have suggested that KGF can increase the radio-resistance of epithelial cells by enhancing DNA repair<sup>332</sup>, altering expression of mediators or antagonists of apoptosis<sup>338</sup>, or by altering the ability of cells to scavenge free radicals<sup>335-337</sup>. However, we were unable to detect any indication for induction of radio-resistance by  $\Delta$ N23-KGF using our *ex vivo* salisphere assay. Instead, we suggest that the expansion of the stem cell pool appears responsible for the observed amelioration of radiation-induced damage to the submandibular gland. We showed that pre-treatment with  $\Delta$ N23-KGF increases the number of progenitor/stem cells leading to a higher absolute number after radiation.

Post-treatment  $\Delta$ N23-KGF can accelerate the proliferation/expansion of the fraction of progenitor/stem cells that survived the radiation, and hence further stimulate the effect of pre-treatment  $\Delta$ N23-KGF.

$\Delta$ N23-KGF enhanced BrdU labelling in cells of acinar and duct compartments of the submandibular gland which suggest that all cell types are proliferating. However, acinar cells do not express the FGFR2-IIIb receptor, and are therefore not likely to be stimulated by  $\Delta$ N23-KGF. Differentiation from intercalated duct cells into acinar cells has been shown to occur in submandibular glands of mice<sup>128</sup>, rats<sup>122,129</sup> and humans<sup>339</sup>. Therefore, the labelling and enhanced number of BrdU-positive acinar cells is probably caused by proliferation and subsequent differentiation of intercalated duct cells. After  $\Delta$ N23-KGF stimulation, excretory and intercalated duct cells, both of which do express the  $\Delta$ N23-KGF receptors, rapidly increased in number, resulting in elongation of excretory ducts. Interestingly, during normal aging these ducts decrease in length<sup>128</sup>. The current study shows that after irradiation the number of surviving salivary gland stem/progenitor cells can be doubled by a pre-treatment with  $\Delta$ N23-KGF.

The capacity of surviving stem/progenitor cells to (partly) repopulate the gland after stimulation with  $\Delta$ N23-KGF provides an interesting opportunity for novel targeted therapy. For future clinical use, a potential issue of concern is the possibility that  $\Delta$ N23-KGF may stimulate tumor proliferation.

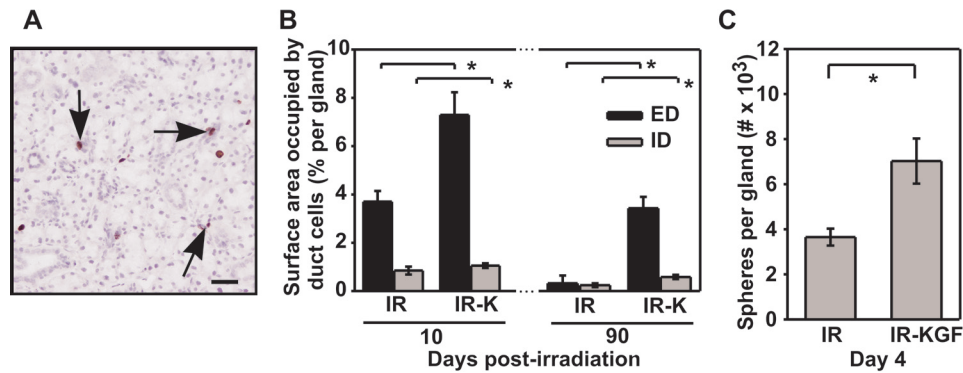
However, e.g. malignant hematopoietic cells are unresponsive to  $\Delta$ N23-KGF<sup>340</sup>, and strikingly  $\Delta$ N23-KGF is now used in phase III trials to prevent chemo/radiotherapy-induced oral mucositis in patients with hematopoietic malignancies<sup>340</sup>. Furthermore, head and neck squamous carcinoma cell lines expressing FGFR2-IIIb receptors, did not show an *in vitro* growth advantage or alteration in radiation sensitivity relative to normal nasal epithelial cells upon  $\Delta$ N23-KGF stimulation<sup>341,342</sup>. This may indicate that therapeutically effective doses of  $\Delta$ N23-KGF may not stimulate head and neck tumor cell growth. However, this issue needs to be further explored carefully.

Local intraglandular administration of  $\Delta$ N23-KGF may be another promising tool in clinical use, for example by using intraductal injections or  $\Delta$ N23-KGF-releasing microspheres, similar to EGF/bFGF-releasing biodegradable microspheres that have been used in parotid glands<sup>343</sup>. Potentially, both hyposalivation and oral mucositis might be prevented simultaneously when  $\Delta$ N23-KGF is administrated systemically.

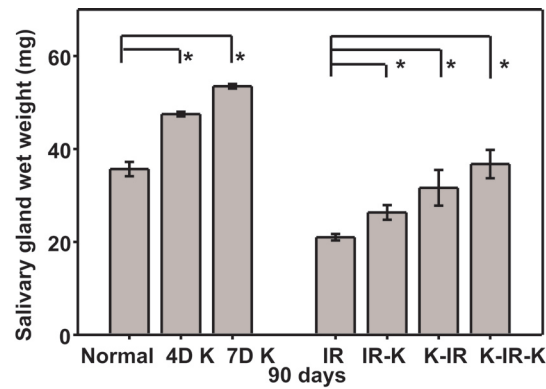
Our study provides the first evidence that *in vivo* induction of expansion and differentiation of stem/progenitor cells by  $\Delta$ N23-KGF protects salivary glands against radiation damage. Hence,  $\Delta$ N23-KGF is a promising therapeutic modality to prevent radiation-induced gland dysfunction in head and neck cancer patients.

ACKNOWLEDGMENTS

We thank Dr. P. van Luijk for his assistance on the statistical analysis of the data.



**FIGURE 5. ΔN23-KGF STIMULATES SURVIVING DUCT CELLS TO PROLIFERATE AFTER IRRADIATION.** (A) BrdU retaining intercalated duct (ID) cells (arrows) were present in glands from IR-K (see Figure 1) treated mice, 24 hours after the last ΔN23-KGF/BrdU injection. (B) The surface area occupied in by excretory duct (ED) and ID cells, expressed as percentage per gland, was significantly increased in IR-K mice compared to IR mice both at 10 and 90 days post-radiation. (C) Significant more salispheres could be recovered from glands from IR-K mice compared to IR treated glands.



**SUPPLEMENTAL FIGURE 1. INFLUENCES OF ΔN23-KGF ON GLAND WET WEIGHT.** Salivary gland wet weight was significantly increased in IR-K, K-IR and K-IR-K treated mice compared to untreated irradiated mice (IR), 90 days post-radiation. Glands from 4D and 7D treated mice had a significantly higher gland weight compared to normal. \*,  $P < 0.05$ ,  $N \geq 3$ .

MATERIALS AND METHODS

ANIMALS

Female C57BL/6 mice, 8-12 weeks old, were purchased from Harlan (Horst, NL). The mice were kept under clean conventional conditions, and fed *ad libitum* with food pellets (RMH-B, Hope Farms B.V., Woerden, The Netherlands) and acidified tap water (pH = 2.8). All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

IRRADIATION OF THE SALIVARY GLANDS

Salivary glands were locally irradiated with a single dose of 10; 12.5; 15; 17.5 or 20 Gy of X-rays (Philips CMG 41 X, 200kV, 10mA, 5 Gy/minute). Mice were protected from off-target radiation by a lead shield. These radiation doses are known to induce sufficient damage without compromising the general health of the animals.

ΔN23-KGF AND BrdU ADMINISTRATION

ΔN23-KGF (5mg/kg/day, Amgen, Thousand Oaks, CA) was subcutaneously administered prior to or/and directly after irradiation according to the scheme in Figure 1. Unirradiated mice received ΔN23-KGF for 4 or 7 consecutive days. To determine the effect of ΔN23-KGF on cell proliferation, BrdU (i.p. 50mg/kg) was administered together with ΔN23-KGF treatment, when appropriate. Twenty-four hours after the last ΔN23-KGF/BrdU injection, mice were sacrificed and glands were collected for further investigation.

SALIVA COLLECTION

At 30, 60 and 90 days post-irradiation saliva flow rate was determined. Animals were placed in a restraining device <sup>173</sup> and saliva was collected for 15 minutes after pilocarpine injection (2 mg/kg, s.c.). The saliva volume was determined gravimetrically, assuming a density of 1 g/mL for saliva.

IMMUNOHISTOCHEMICAL PROCESSING

After extirpation, the submandibular glands were weighed and incubated for 30 hours at 4° C in 4% buffered formaldehyde. Following dehydration, the tissue was embedded in paraffin. Five-micrometer sections were analyzed for acinar cells using PAS (Periodic Acid Schiff's base) staining. Ductal cells were identified using an anti-CK7 (Monosan, Burlingame, CA; MON3007) antibody, the receptor for KGF (KGFR) was detected using an anti-FGFR2IIIb (RnD Systems, Minneapolis, MN; MAB7161) antibody after a trypsin pre-treatment, and proliferation was assessed by BrdU presence using anti-BrdU antibodies (Abcam, Cambridge, UK; ab6326) (1/500 1hr) after citrate treatment. Secondary anti-rat biotine antibodies, (Elite ABC-kit, Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB) were used to detect the expression. In addition, nuclear staining was performed (hematoxylin).

QUANTIFICATION OF ACINAR AND DUCTAL CELLS IN SALIVARY GLANDS

Tissue sections of submandibular glands were analyzed using bright field microscopy (Olympus CX40, Germany) under 400x magnification evaluating 100 squares of 0.25 mm<sup>2</sup> each. The percentage of surface area occupied by acinar cells was counted from two different sections (top-middle) of each submandibular gland.



Quantification of the different duct compartments (excretory, striated, and intercalated ductal cells) was conducted by using the AnalySIS program (Olympus, Soft Imaging System, Münster, Germany) by measuring the area occupied by the different duct compartments. In further data processing, the percentage of the surface area of the ducts and acinar cells per gland was calculated.

#### DETERMINATION OF STEM/PROGENITOR CELL NUMBER

Submandibular glands were extirpated and processed for cell isolation as described before <sup>220</sup>. Salivary gland cells were plated in a defined medium of DMEM/F-12 (Gibco, Carlsbad, CA; 41966-029, 21765-029), penicillin, streptomycin, glutamax, EGF (20 ng/mL), FGF-2 (20 ng/mL), N<sub>2</sub> (1/100), insulin (10 µg/mL) and dexamethasone (1 µM) <sup>220</sup>. All growth factors were purchased from Sigma-Aldrich (St. Louis, MO), except for N<sub>2</sub> (Gibco, Carlsbad, CA). After 3 days of culture, spheres were counted and recalculated as a percentage per plated cells.

For flow cytometric analysis of c-Kit<sup>+</sup> cells, salispheres were dissociated by 0.05% trypsin-EDTA (Gibco, Carlsbad, CA; 25300) with mechanical use of 26G needles. Cells were incubated for 20 minutes with anti-c-Kit FITC (BD Biosciences Pharmingen-553354) antibodies at 4°C. Cells were analysed on a FACS Calibur Flow Cytometer (Beckton Dickinson) after the addition of Propidium Iodide (2 µg/mL) to select for living cells. For each measurement a minimum of 100,000 events were collected. Data were analyzed using FlowJo (Tree Star, Ashland OR).

#### STATISTICAL ANALYSIS

The results were analyzed using a Mann-Whitney or Student *t*-test. Statistical significance was defined as *P* < 0.05 using SPSS. Numbers represent mean ± SEM.